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Molecular cloning and characterization of a nematode polyprotein antigen/allergen from the human and animal hookworm *Ancylostoma ceylanicum*

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Abstract

Nematodes are unable to synthesize fatty acids *de novo* and must acquire them from the environment or host. It is hypothesized that two unique classes of fatty acid and retinol binding proteins that nematodes produce (fatty acid and retinol binding (FAR) and nematode polyprotein antigen/allergen (NPA)) are used to meet this need. A partial cDNA has been cloned corresponding to four subunits of a putative *Ancylostoma ceylanicum* NPA (AceNPA). The translated amino acid sequence of AceNPA share sequence identity with similar proteins from *Dictyocaulus viviparus*, *Ascaris suum*, and *Ostertagia ostertagi*. Immunoblot experiments using a polyclonal anti-AceNPA IgG revealed proteins corresponding to the expected sizes of single, as well as two or three un-cleaved NPA subunits in adult excretory/secretory proteins and soluble adult worm extracts. Immunohistochemistry experiments localize AceNPA to the cuticle and pseudocoelomic space, suggesting a role in hookworm biology that is distinct from what has previously been defined for other hookworm lipid binding proteins. A single recombinant subunit of AceNPA (rAceNPAb) demonstrated binding *in vitro* to fluorescent fatty acids DAUDA, cis-parinaric acid, as well as retinol, at equilibrium dissociation constants in the low micromolar range. Further, *in vitro* data reveal that rAceNPAb binds fatty acids with chain lengths of C12–C22, with the greatest affinities for arachidonic, linoleic (C18), and eicosapentaenoic (C20) acids.

Keywords

Fatty acid; Nematodes; *Ancylostoma ceylanicum*

1. Introduction

Nematodes require fatty acids and retinol for the biosynthesis of most classes of lipids and glycoproteins [1]. These lipids, along with free fatty acids, are integral components of the

nematode cuticle [2,3], hypodermis, eggshell, male and female gamete, and cell membranes of each life-stage of the worm. Free fatty acids and retinol have also been implicated in nematode growth, development, and neurological processes. In *Caenorhabditis elegans*, the long chain polyunsaturated fatty acids ARA (arachidonic acid) and DHA (docosahexaenoic acid) are involved in the neurotransmission necessary for locomotion and egg laying [4], and ARA is involved in the signaling pathway of sperm recruitment to the spermatheca [5]. The shorter (18 carbon) polyunsaturated fatty acids gamma-linolenic acid and stearidonic acid are required for *C. elegans* immunity to *Pseudomonas aeruginosa* [6], suggesting that polyunsaturated fatty acids are necessary for multiple biological functions in nematodes.

Parasitic helminths are unable to synthesize these required fatty acids and retinol *de novo* and must acquire them from the environment or host to meet various development needs [7–10]. For example, multiple life cycle stages of *Brugia malayi* metabolize exogenous radio-labeled retinoic acid, with the greatest label accumulation seen in the cellular portions of early and late developing embryos [11]. Culturing adult *Litomosoides carinii* and other filarial worms with synthetic retinoids leads to reduced adult worm motility and the suppression of the release of microfilaria [12], and also inhibits the molting of *Onchocerca lienalis* L₃ [13].

To date, at least two classes of fatty acid binding proteins have been identified in parasitic nematodes: the nematode polyprotein antigens/allergens (NPA) and the fatty acid and retinol binding (FAR) proteins [14–16]. The NPA proteins are synthesized as polyproteins containing 10 or more nearly identical subunits. The polyprotein is post-transcriptionally cleaved at a consensus processing site into single subunits (~15 kDa) that bind both fatty acids and retinol in the micromolar to sub-micromolar range [17–23], similar to other small lipid transporters [24,25]. However, the individual subunits have an α -helix rich structure, making them structurally different from the lipid transporters found in vertebrates. These smaller subunits are processed and secreted from worms into the host and surrounding environment [26–31].

Due to the requirement of exogenous fatty acids by the parasites, host fatty acid levels may influence pathogenesis of disease caused by parasitic nematodes. For example, manipulating the percentages of EPA (eicosapentaenoic acid), DHA, and docosapentaenoic acid in the gut mucosa of calves alters the number of immature intestinal worms recovered following infection with *Ostertagia ostertagi* and *Cooperia oncophora* [32]. Retinol depletion of cotton rats infected with *L. carinii* retards the development of microfilaria in the uteri of female worms [33].

Host IgE and IgG4 responses directed against ABA-1, the prototypical NPA from *Ascaris suum*, correlate with immunity to the human roundworm *A. lumbricoides* [26,34,35]. In veterinary disease, vaccination of calves with the *O. ostertagi* NPA reduces both pathology and egg output [36]. Recent work in the hamster model of *Ancylostoma ceylanicum* infection showed that animals vaccinated orally with the hookworm FAR protein rAceFAR-1 exhibited a statistically significant (40–47%) reduction in intestinal worm burden compared to controls [37]. These observations suggest a role for nematode fatty acid

binding proteins in disease pathogenesis, and as such, make them potential targets for drug and vaccine development.

We report here the molecular cloning and *in vitro* characterization of a cDNA corresponding to a nematode polyprotein antigen/allergen (NPA) from the human and animal hookworm *A. ceylanicum*. Based on its immunolocalization and specific fatty acid binding profile, we propose that the AceNPA protein may play a role in parasite development and uptake of host fatty acids.

2. Materials and methods

2.1. Hookworm life cycle and parasite antigens

The *A. ceylanicum* life cycle was maintained in hamsters as previously described [38]. Adult worms were manually harvested from the small intestines at day 20 post-infection (PI) and used to prepare soluble hookworm extracts (HEX) and excretory/secretory (ES) products [39]. Protein content was determined by using abicinchoninic acid protein assay system (BCA) (Pierce Chemical Co., Rockford, IL.) with a bovine serum albumin standard curve. Eggs and newly hatched larvae (L1) were collected from adult females cultured overnight in RPMI/50% fetal calf serum (FCS) [40,41]. The animal research protocols employed in this study were approved by the Yale University Animal Care and Use Committee and complied with all relevant federal guidelines.

2.2. Cloning of the AceNPA cDNA

Thirty live adult *A. ceylanicum* (equal numbers of males and females) were suspended in Trizol (Invitrogen), and total RNA was extracted according to manufacturer's suggestions. First strand cDNA [39,42] was combined with a gene specific forward oligonucleotide primer (AceNPAF5) and reverse primer (AceNPAR5) were designed based on the aligned consensus sequence of the NPA orthologue from *O. ostertagi* (NCBI Accession number Z46800) and the related dog hookworm *Ancylostoma caninum* (NCBI Accession number BQ667055). After amplification, the product from this reaction was ligated into the pCR2.1 plasmid vector (Invitrogen) and One Shot *Escherichia coli* INVαF⁺ cells (Invitrogen) were transformed. Insert-positive clones were sent to the William Keck Biotechnology Facility at Yale University for sequencing. The resulting sequence was used to make an *A. ceylanicum* gene specific forward primer (AceNPAF6) used with oligo dT to amplify the complete 3' end of AceNPA.

The reverse primer (AceNPAR5) was used with the vector primer corresponding to the T3 promoter (T3P) to amplify the 5' region of AceNPA from an adult *A. ceylanicum* cDNA library (Lambda zap; Strategene). The nucleotide and translated amino acid sequences were analyzed for similarity to other known NPA genes and proteins using the BLAST algorithm available through the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/>). Multiple sequence alignment and analyses were carried out using the ClustalW algorithm [43] and were used to generate the partial sequence of AceNPA (3 complete (a–c) and 1 incomplete subunit).

2.3. Expression and purification of recombinant AceNPA

The cDNA corresponding to a predicted b subunit of AceNPA (AceNPAb) was directionally cloned into the pET-28a expression plasmid vector (Novagen, Inc., Madison, WI, USA). The ligated plasmid was used to transform *E. coli* BL21 DE3 cells (Novagen) using the manufacturer's protocol. A single colony containing the insert was used to initiate a liquid culture, and recombinant protein expression was induced by addition of isopropyl- β -D-thiogalactopyranoside (IPTG) to a concentration of 1 mM. The soluble rAceNPAb protein was purified from bacterial lysates by nickel resin affinity chromatography using a Hi-Trap chelating Sepharose column (Amersham Biosciences Corp., Piscataway, NJ, USA) [39,44]. The cDNA for three uncleaved subunits of AceNPA (AceNPAc) was expressed and purified in the same manner.

2.4. Generation of rabbit polyclonal anti-rAceNPAc IgG

A polyclonal antiserum was raised in a rabbit by subcutaneous injection with 100 μ g of purified rAceNPAc in Freund's complete adjuvant, followed by three subsequent injections with 100 μ g of purified rAceNPAc in incomplete Freund's adjuvant at 2 week intervals. The rabbit IgG fraction was purified from serum using a Protein G affinity chromatography column (Amersham Pharmacia Biotech, Upsala, Sweden) [37,45].

2.5. Localization of AceNPA using immunoblot and immunohistochemistry

The sex and lifestage specificity of AceNPA was characterized using immunoblot as previously described [37]. *A. ceylanicum* HEX (2.5 μ g) and ES (3 μ g) prepared from either adult males or females were separated by SDS-PAGE, followed by electroblot transfer to nitrocellulose. After blocking, the membrane was incubated at 4°C overnight with the anti-rAceNPAc IgG (0.5 μ g/ml), and bound antibody was detected using horseradish peroxidase (HRP)-labeled goat anti-rabbit polyclonal IgG (0.5 μ g/ml) (Sigma) with chemiluminescence. Control blots were performed using pre-immune rabbit IgG as the primary antibody.

2.6. Ligand binding experiments

The fatty acid binding profile of rAceNPAb was created using the fluorescent analog 11-((5-dimethylaminonaphthalene-1-sulfonyl)amino) undecanoic acid (DAUDA) and the naturally fluorescent cis-parinaric acid (Molecular Probes) [37,46–48]. Fluorescence emission spectra were recorded at 25°C in a total volume of 200 μ l per well using a Varioskan Flash (Thermo Electron Corporation, Waltham, MA, USA). The fluorescence emission spectra for AceNPAb bound to retinol (Sigma, St. Louis) and cis-parinaric acid (Sigma) were determined in a similar manner. The excitation wavelengths used for DAUDA, retinol, and cis-parinaric acid were 345, 350, and 319, respectively. All fluorescent compounds were stored at –20°C and freshly diluted in ethanol before use.

Competition studies were carried out by monitoring the change in fluorescence intensity at the peak transmission wavelength measured for the rAceNPAb:DAUDA complexes in the presence of a 10-fold excess of various unlabelled fatty acids. The equilibrium dissociation constant (K_d) for rAceNPAb binding to DAUDA was estimated by adding increasing concentrations of rAceNPAb to a solution of 1.0 μ M DAUDA in PBS (total volume of 200

μl). In order to determine the K_d for rAceNPAb:retinol and rAceNPAb:cis-parinaric acid binding, increasing concentrations of fluorescent ligand were added to a 1.0 μM solution of rAceNPAb in PBS. Fluorescence data were normalized to the peak fluorescence intensity and corrected for background fluorescence of the ligand alone at each concentration. Corrected data were then analyzed using the one site saturation model and best fit algorithm ($y = (B_{\max} \cdot X)/(K_d + X)$) contained within the SigmaPlot9 (Systat software) software as previously described [37].

3. Results

3.1. Cloning of the *A. ceylanicum* nematode polyprotein antigen/allergen cDNA and expression of recombinant protein

A partial cDNA was identified in the *A. caninum* EST database that has sequence similarity to other known nematode polyprotein antigen/allergens (NPA) proteins. Primers (AceNPAF5 and AceNPAR5) were designed from areas of highest sequence similarity among the known sequences from *A. caninum*, *A. suum* and *O. ostertagi*. Using PCR, we amplified an *A. ceylanicum* cDNA whose translated amino acid sequence showed similarity to these NPA proteins [18,20,36]. This sequence was then used to design a specific forward primer (AceNPAF6) for use with oligo dT to amplify the true 3' end of the cDNA. AceNPAR5 was used with a vector primer (T3P) to amplify the 5' end from the *A. ceylanicum* lambda zap library. Using this approach three full and one partial subunit of the putative *A. ceylanicum* nematode polyprotein allergen/antigen (*AceNPA*) were amplified.

Translation of this partial cDNA reveals 30–47% amino acid sequence identity with *O. ostertagi*, *Dictyocaulus viviparus*, and *A. suum* (Fig. 1). The translated sequence also has the conserved Lys/Arg-Xaa-Lys/Arg-Arg cleavage site for processing enzymes [49,50] (Fig. 1A), suggesting that the AceNPA polyprotein is processed into individual subunits in a manner similar to other nematode orthologues. The three subunits of *AceNPA* have an additional Asp-His following the cleavage site as has been described for some subunits of the *D. viviparus* NPA [20] (DvA-1). This observation is different from the NPA proteins from *O. ostertagi* and *A. suum* (ABA-1), suggesting that *AceNPA* may be more closely related to *DvA-1*. Computer based secondary structure analysis [51,52] of the three subunits individually predicts a strong α-helix and coiled coil structure for each (Fig. 1A), similar to the other known NPA proteins [20,22,23,29,36]. The three cloned subunits of AceNPA have extensive amino acid heterogeneity (Fig. 1B), with 66–67% amino acid identity. This is distinct from what has been reported for *A. suum* and *O. ostertagi*, but is similar to what is seen with *D. viviparus*, again suggesting that the *A. ceylanicum* NPA is most closely related to DvA-1.

The cDNA corresponding to a single complete subunit was directionally cloned into the pET11a prokaryotic expression vector. The induced cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting using a monoclonal antibody against the C-terminal fusion protein His tag. The rAceNPAb protein was purified from cell lysates using nickel resin affinity chromatography and Coomassie staining was performed to assess the degree of protein purity (>80%, data not shown). Immuno-staining reveals a single band at the expected size of rAceNPA (15,413.6 Da), as well as a band at the

predicted size of a dimer of rAceNPA. The cDNA corresponding to three contiguous subunits (rAceNPACA) was also cloned in frame into pET11 and the rAceNPACA protein purified from cell lysates using nickel resin affinity chromatography. This protein was used to generate a polyclonal anti-rAceNPACA antibody in rabbits. Specificity of the antibody was assessed using Western blot analysis of immunizing and unrelated recombinant proteins (data not shown).

3.2. Life cycle stage, sex, and tissue specific detection of AceNPA

Immunoblots of native hookworm protein extracts were probed with the rabbit polyclonal anti-rAceNPACA IgG. A band consistent with a single cleaved subunit (~15 kDa) can be seen in both male and female *A. ceylanicum* HEX (Fig. 2A) as well as male and female ES, indicating that the protein is translated and secreted by both sexes. This finding is similar to data from other NPA proteins, which are detected as single subunits in the ES of adult worms and larvae [20,29,36]. The anti-AceNPACA antibody also recognizes a band of the same size in soluble extracts from L₃ larvae (LEX), indicating that the protein is present in almost all life-stages. Interestingly there is very little protein detected in eggs, an expression pattern that is distinct from what we have previously seen with AceFAR-1 [37]. Higher molecular weight bands that correspond to the size expected for two un-cleaved subunits can also be seen in the HEX lanes, while bands that corresponded to the predicted size of three un-cleaved subunits can be seen in the ES samples for both male and female, suggesting that incompletely cleaved forms of the protein may be released by the worm, a pattern that also occurs in *D. viviparus* [29].

In order to further define the role of AceNPA in *A. ceylanicum* biology, sagittal sections of adult *A. ceylanicum* were probed with the anti-AceNPACA IgG. AceNPA localized to the pseudocoelomic cavity (PC) and hypodermis/cuticle (H/C) (Fig. 2B) of both males and females, as well as the testes (T) of adult males (Fig. 2B). This finding is similar to what has been observed for *Ascaridia galli* [23], where AgNPA-1 localized to the pseudocoelomic cavity (particularly around the ovaries), *A. suum* [23], where the protein localizes to the inner hypodermis, and *O. ostertagi* [36], where the protein localizes to the hypodermis/cuticle of L4 and adult worms. The localization of AceNPA to the testes of males is unique among nematodes studied to date, but similar to the localization pattern of AceFAR-1 [37].

3.3. Ligand specificity and binding kinetics of rAceNPA

The ligand specificity of recombinant AceNPA binding was characterized using a modified fluorescent plate based assay [53]. Under the conditions of this assay rAceNPAb bound DAUDA with the peak of fluorescence emission occurring at 496 nm (Fig. 3A). This degree of blue shift in emission by DAUDA (from 543 nm in buffer alone to 496 nm) indicates that the fluorophore has been taken into a highly non-polar rAceNPAb binding site [25,54]. This blue shift was greater than the blue shift seen with bovine serum albumin [55], and within the range of shifts seen for other NPA proteins [17], but less dramatic than the shift seen with rAceFAR-1 [37] and ABA-1[22], suggesting that there may be structural differences between the DAUDA binding sites of rAceNPAb and rAceFAR-1. The addition of a 10-fold excess of oleic acid is able to reduce both the fluorescence intensity, and the peak of

emission near to the level seen with DAUDA alone suggesting that DAUDA is binding at the fatty acid binding site.

The fatty acid binding specificity of rAceNPAb was further elucidated by measuring the degree of displacement of DAUDA (*i.e.* the reduction in fluorescence intensity at 496 nm) in the presence of an excess of various non-fluorescent fatty acids. It was demonstrated that rAceNPAb binds saturated and unsaturated fatty acids with chain lengths C_{12} – C_{22} with the maximal reduction of fluorescence (Fig. 3B) by the monounsaturated oleic acid (C_{18}) and the polyunsaturated arachidonic acid (C_{20}). Interestingly, competition with EPA (a polyunsaturated C_{20}) and LIN (a polyunsaturated C_{18}) reduces fluorescence intensity 80 and 75% (respectively), suggesting relatively high affinity binding by rAceNPAb. This contrasts what we have previously reported for rAceFAR-1 [37], suggesting that these two proteins have different mechanisms of action. Cholesterol (CHO) did not reduce fluorescence intensity, suggesting that, as is true with rAceFAR-1 and other NPA and FAR proteins, it is not bound by rAceNPAb.

The rAceNPAb:DAUDA equilibrium dissociation constant (K_d) was calculated using data from *in vitro* titration experiments. Fig. 3C shows a titration curve (corrected for the background fluorescence of DAUDA), which predicts a K_d of 1.93×10^{-6} for the rAceNPAb:DAUDA interaction, which is higher than what has been reported for other NPA proteins [17,56], but is within the range of reported equilibrium dissociation constants for soluble transporters. We found that rAceNPAb also binds *cis*-parinaric acid ($K_d = 5.5 \times 10^{-7}$, Fig. 4A and B) and retinol ($K_d = 2.26 \times 10^{-6}$, Fig. 4C and D). The displacement of retinol by oleic acid indicates that either AceNPAb has a single binding site for retinol and fatty acids, or the binding sites are overlapping or interfering.

4. Discussion

Because parasitic nematodes are unable to synthesize fatty acids and retinol *de novo*, they must acquire these essential compounds from the environment or host [7–10]. There is evidence to suggest that altering the relative amounts or timing of exposure to specific fatty acids may influence the growth and development of free living and parasitic nematodes. For example, *B. malayi* worms take up exogenous radio-labeled retinoic acid, with the greatest label accumulation seen in the cellular portions of early and late developing embryos [11], suggesting that retinoic acid may be involved in parasite development. The culturing of adult *L. carinii* and other filarial worms with synthetic retinoids leads to reduced adult worm motility and the suppression of the release of microfilaria [12]. Synthetic retinoids also inhibit the molting of *O. lienalis* L₃ [13], again suggesting that retinol is involved in embryonic and larval development in nematodes.

Altering host fatty acid and retinol composition may also influence disease pathogenesis caused by parasitic nematodes. For example, retinol depletion in *L. carinii* infected cotton rats retards the development of microfilaria in the uteri of female worms [33], while increasing the percentages of EPA (eicosapentaenoic acid), DHA, and docosapentaenoic acid in the gut mucosa of calves leads to higher number of immature intestinal worms following infection with *O. ostertagi* and *C. oncophora* [32]. Likewise, targeting fatty acid

uptake by parasitic nematodes using a vaccine strategy has been shown to reduce the intensity of hookworm infection in an animal model of ancylostomiasis [37].

We describe here the molecular cloning and characterization of a novel nematode polyprotein antigen/allergen (NPA) from the human and animal hookworm *A. ceylanicum*. The cloned subunits of *A. ceylanicum* *AceNPA* have extensive sequence diversity (when aligned with each other), which is distinct from the *Ascaris* NPAs, which have only two related subunits, but similar to what has been reported for *D. viviparous* [29]. This, along with the similarity in amino acid sequence surrounding the cleavage sites of *AceNPA* and *DvA-1*, suggests that among the cloned NPA sequences, *AceNPA* and *DvA-1* may be the most closely related. Similar to known NPA proteins, the individual subunits of the cloned *AceNPA* cDNA have both an α -helix rich and a coiled coil structure. This indicates that, despite amino acid diversity, both between subunits and across species, the secondary structure of NPA proteins is conserved.

Immunoblot and immunolocalization analysis revealed sex and stage-specific differences in native *AceNPA* protein expression. *AceNPA* protein is detectable in all life-stages, although protein extracts from eggs have the least immunoreactivity, while third stage larvae (L₃), and both male and female HEX have the greatest immunoreactivity. This pattern is distinct from what was observed for *AceFAR-1*, where eggs have the greatest transcript abundance and protein immunoreactivity and only female ES has detectable amounts of *AceFAR-1* [37]. This expression difference suggests that *AceNPA* and *AceFAR-1* have distinct functions in the biology of *A. ceylanicum*. Probing of immunoblots with a polyclonal IgG directed against r*AceNPACa* reveals protein bands corresponding to single subunits, as well as bands the size of two or three uncleaved subunits, in protein extracts and ES of *A. ceylanicum*, suggesting that multiple forms of *AceNPA* are processed and released by various stages of the parasite. These larger bands could be either partially cleaved subunits, or could represent unidentified subunits without cleavage sites, as have been identified in *Dirofilaria immitis* [28,57] and *D. viviparus* [29]. These forms may have functions in pathogenesis that are distinct from those of single subunits, since they are released into the host.

Immunohistochemistry experiments revealed sex specific differences in the localization of *AceNPA*. The localization to the pseudocoelomic cavity (PC) and the hypodermis/cuticle in both males and females is not surprising, as this has been reported for several NPA species [22,23,28,58]. What appears unique to *A. ceylanicum* is the localization of *AceNPA* to multiple layers of the testes in males. The lack of signal within the spermatheca in the female (Fig. 2B) suggests that the antibody is bound to the testes and not to the sperm within the testes [45]. To our knowledge, this has not been reported for any other NPA protein. This is again distinct from the localization pattern we have reported for *AceFAR-1*, suggesting that the two fatty acid binding proteins have unique roles in *A. ceylanicum* pathobiology.

The localization of *AceNPA* to the hypodermis/cuticle raises two possible roles for *AceNPA* in *A. ceylanicum*. The first possibility is that this protein is involved in the acquisition/transport of specific fatty acids necessary to maintain the integrity of the cuticle. The second

possibility is that AceNPA is directly interacting with molecules at the site of hookworm attachment. This would be similar to what has previously been theorized for AceKI, a secreted serine protease inhibitor that also localizes to the sub-cuticle [44].

Similar to rAceFAR-1, rAceNPAb binds fatty acids with chain lengths C₁₂–C₂₂ although there are differences in the relative affinities of rAceNPAb for these fatty acids. The DAUDA displacement assay indicates that rAceNPAb has a relatively high affinity for EPA and LIN (20 and 25%, respectively, of the fluorescence intensity of DAUDA alone) compared to rAceFAR-1 (60 and 80%, respectively, of the fluorescence intensity of DAUDA alone [37]). This relative affinity difference provides more evidence that AceNPA and AceFAR-1 have different roles in the biology of *A. ceylanicum*. The nature of this functional difference has not yet been elucidated, but the circumstantial evidence suggests that AceNPA is involved in the acquisition of specific fatty acids necessary to fulfill the metabolic requirements of the male reproductive system, whereas we believe that AceFAR-1 is involved in egg generation in the female reproductive tract [37]. Considering the sequence diversity of the three cloned AceNPA subunits it is possible that the other subunits have different relative affinities for the tested fatty acids. In order to address this possibility, future experiments will evaluate recombinant versions of additional subunits to determine specific fatty acid binding profiles.

In summary, the studies reported here suggest that the two known lipid binding proteins from the human and animal hookworm *A. ceylanicum* likely fulfill distinct roles in hookworm development (from egg to blood feeding adult) and metabolism. While the definitive roles of these proteins in hookworm pathogenesis remains to be elucidated, the essential functions of these proteins combined with their unique structures make them attractive targets for vaccines and therapeutics designed to reduce hookworm associated pathology.

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Fig. 2.

The sex and developmental stage specific expression of the AceNPA protein. (A) Polyclonal anti-rAceNPACA IgG was used to probe immunoblots containing sex-specific (F: female; M: male) soluble adult hookworm extracts (HEX, 2.5 μ g) and excretory/secretory proteins (ES, 3 μ g), as well as soluble extracts from third stage larvae (L3, 2.5 μ g) and total protein extracts from eggs (E). (B) Cryosections of adult female (top two panels) and male (bottom two panels) hookworms were probed with the anti-rAceNPACA IgG (center) or Pre-immune IgG (right). Images suggest that native AceNPA is present in the hypodermis/cuticle (H/C), and pseudocoelomic cavity (PC) of females, and the hypodermis (H/C), pseudocoelomic cavity (PC), and testes (T) of males.

Fig. 3.

In vitro binding specificity of rAceNPAb. (A) Fluorescence emission spectra ($\lambda_{\text{exc}} = 345$ nm) of $0.8 \mu\text{M}$ DAUDA, $3 \mu\text{M}$ rAceNPAb, and a mixture of both compounds. (B) Effect of a 10-fold excess of individual unlabelled fatty acids on the fluorescence intensity of the DAUDA-rAceNPAb complex (measured at 488 nm). *Indicates a statistically significant ($P < 0.01$) difference in mean signal intensity compared to DAUDA control group, as analyzed using ANOVA and Dunnett Multiple Comparison test ($n = 3$). *Abbreviations:* C12, dodecanoic acid; C14, myristic acid; C15, pentadecanoic acid; C16, palmitic acid; C17, heptadecanoic acid; C18, oleic acid; C22, docosahexaenoic acid; EPA, eicosapentaenoic acid; ARA, arachidonic acid; LIN, linoleic acid; CHO, cholesterol. (C) Change in relative fluorescence intensity (488 nm) of DAUDA ($1.0 \mu\text{M}$) in the presence of increasing concentrations of rAceNPAb. The best-fit curve was used to determine the equilibrium dissociation constant (K_d) for the DAUDA:rAceNPAb interaction.

Fig. 4.

In vitro binding of cis-parinaric and retinol by rAceNPAb. (A) Fluorescence emission spectra ($\lambda_{\text{exc}} = 319 \text{ nm}$) of $0.8 \mu\text{M}$ cis-parinaric acid alone, or in combination with $3 \mu\text{M}$ rAceNPAb. The displacement effect following addition of $10 \mu\text{M}$ oleic acid to the preformed cis-parinaric acid:rAceNPAb complex is also shown. (B) Change in relative fluorescence intensity ($\lambda_{\text{exc}} = 319 \text{ nm}$) of $1.0 \mu\text{M}$ rAceNPAb in the presence of increasing concentrations of cis-parinaric acid. The curve was used to derive the equilibrium dissociation constant, K_d for the cis-parinaric acid:rAceNPAb interaction. (C) Fluorescence emission spectra data ($\lambda_{\text{exc}} = 350 \text{ nm}$) for rAceFAR-1 binding to $0.8 \mu\text{M}$ retinol. The displacement effect following addition of $10 \mu\text{M}$ oleic acid to the preformed retinol:rAceNPAb complex is also shown. (D) Change in relative fluorescence intensity ($\lambda_{\text{exc}} = 319 \text{ nm}$) of $1.0 \mu\text{M}$ rAceNPAb in the presence of increasing concentrations of retinol. The curve was used to derive the K_d for the retinol:rAceNPAb interaction.